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(54) Methods for the use of spermidine/spermine N'-acetyltransferase as a progonostic indicator and/or a tumor response marker.

(57) Disclosed is a method that relates to the measurement of determinants related to the in-vivo induction of spermidine/ spermine N[1]-acetyltransferase (SSAT) , subsequent to treatment with polyamine analogs that induce SSAT such as bis-ethyl spermine analogs, in tumor types responsive to this class of analogs. The method comprises the measurement of one or more SSAT-specific determinants that include SSAT enzyme activity, SSAT enzyme protein, and SSAT m-RNA transcripts. Alternatively, other determinants related to the SSAT induction may be measured. Such determinants include SSAT co-factor acetylCoenzyme A, and SSAT products N[1]-acetylspermidine and N[1]-acetylspermine. Measurements of these determinants may be useful as prognostic indicia and tumor response markers to evaluate the clinical effectiveness of anticancer agents comprising polyamine analogs, such as bis-ethyl spermine analogs, that induce SSAT activity.

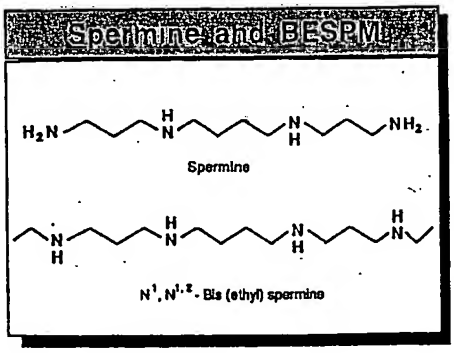

Figure 1.

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1. Background of the Invention:

1.1 Field of the Invention

The present invention relates generally to the detection of enzyme levels that are affected by a specific class of cancer chemotherapeutic agents, and more particularly, to the use of the enzyme spermidine/spermine N^1-acetyltransferase as a prognostic indicator and/or tumor response marker to facilitate clinical use of the class of anticancer agents that effect induction of the enzyme.

1.2 Description of the Background and Related Art

The biological polyamines,putresine, spermidine and spermine are natural components of all mammalian cell types and are known to be essential for cell growth. While their precise role in supporting cell growth is uncertain, it is believed to involve interaction with nucleic acids. These substances and their key biosynthetic enzymes, ornithine and S-adenosylmethionine decarboxylase (ODC and SAMDC, respectively), are increased in neoplastic tissues. Therefore, polyamine biosynthesis has been a targeted in the development of experimental anticancer strategies.

Because inhibitors of polyamine biosynthetic enzymes lower the polyamine content of tumor cells, they are being evaluated for use as anticancer agents. Thus far, these inhibitors have not lead to clinically effective anticancer agents. One of the problems confronting their use is that as soon as polyamine pools are lowered, the key biosynthetic enzymes ODC and SAMDC undergo a compensatory increase in activity (Porter and Bergeron, 1988, in Advances in Enzyme Regulation, pp.57-79, Pergamon Press). Thus, the desired effect of the enzyme inhibitors (i.e. inhibiting cell growth by polyamine pool depletion) is circumvented by one or both of these enzyme responses.

Based on the observation that increases in the polyamine pools suppress ODC and SAMDC activities, an alternative approach has been devised (Porter and Bergeron, 1988, in Advances in Enzyme Regulation, pp.57-79, Pergamon Press). It proposes to identify polyamine analogs which behaves like the natural polyamines in down-regulating ODC and SAMDC, but which lack the ability to perform in functions required for cell growth. N^1, N^{12}-bis(ethyl)spermine (BESPM), a N-bis(ethyl) analog of spermine,has served as a model compound for this strategy. A comparison of the chemical structures of spermine and BESPM is shown in Figure 1. BESPM was found by in-vitro studies to rapidly suppress ODC and SAMDC, deplete natural polyamine pools, and inhibit cell growth at 1-10 uM (Porter et al., 1987, Cancer Res. 47:2821-2825). In addition, BESPM suppresses polyamine uptake (Byers and Pegg, 1990, J. Physiol. 142:460-467; and Kramer et al. Proc. Am. Assoc. Can. Res. 32:404, 1991), and thus minimizes the ability of tumor cells to meet their polyamine requirement by taking them up from their environment. The potential for polyamine analogs as effective cheniotherapeutic agents is evidenced by potent tumor activity against several melanoma cell lines in-vitro (Porter et al.,1991, Cancer Research, 51:3715-3720; Shappell et al., 1992, Anticancer Res., in press) and against MALME-3 human melanoma tumors growing as xenografts in athymic mice, exhibited by bis-ethyl spermine analogs (Bernacki et al., May 1992, Can. Res., in press).

Additional in-vitro studies show that BESPM also causes an profoundly large induction of the polyamine metabolizing enzyme spermidine/spermine N^1-acetyltransferase (SSAT) in certain human carcinoma cell lines. The following is a list of relevant prior art along with a brief description of each:

Libby et al. (Arch. Biochem. Biophys. 284: 238-244, 1991) and Casero et al. (Biochem. J., 270: 615-620) describe isolation and partial characterization of human SSAT protein from BESPM-treated cell lines. Casero et al. report that the cytotoxic response of the NCI H157 human large cell lung carcinoma cell line to exposure to BESPM was associated with a high induction of SSAT in-vitro.

Porter et al. (1991, Cancer Res., 51:3715-3720) describe the extreme induction of SSAT levels after treatment of human melanoma cell lines (MALME-3) in-vitro with BESPM and a lesser degree of induction with other bis-ethyl spermine analogs. Between two melanoma cell lines, the high induction of SSAT activity was suggested to correlate with the growth sensitivity in-vitro to the bis-ethyl spermine analogs.

Each of the above cited references discloses a high induction in the levels of SSAT after treatment of specific human tumor cell lines in-vitro with bis-ethyl spermine analogs. However, there is a need to establish a correlation between SSAT levels and tumor growth responses in animals. In particular, none of the above references demonstrate the induction of SSAT levels following treatment of tumor-bearing animals, i.e. in-vivo, nor do the references disclose correlation of tumor growth sensitivity to bis-ethyl spermine analogs with in-

duced SSAT levels in-vivo. First, it is not known from the prior art whether polyamine analogs analogs are vulnerable to problems related to stability, uptake, and antiproliferative potency in-vivo.

Second, it is not known if the high induction of SSAT would occur in-vivo because of uncertainties related problems related to the analog itself (see above) and to the stability of SSAT-specific m-RNA intracellularly and the stability of the enzyme itself. Because the analogs cause a number of cellular events in in-vitro systems (i.e. suppression of ODC/SAMDC, depletion of polyamine pools, depletion of mitochondrial DNA, and possibly other DNA-related effects), it is important to demonstrate that SSAT induction in-vivo is prominent among the events and that it may be causally related to or indicative of anti-tumor activity. Because an event occurs in-vitro does not necessarily mean that the event occurs in-vivo. For instance, suppression of ODC and SAMDC is well recognized as an in-vitro effect of bis-ethyl spermine analogs. However, recent studies (Porter et al., submitted) show that this effect does not occur in-vivo.

Moreover, it must be demonstrated that SSAT induction in-vivo occurs selectively in tumor tissue relative to various normal tissues. Its potential usefulness as a tumor marker, and as a determinant of drug action, is highly dependent upon induction of enzyme levels selectively in tumor cells. The prior art discloses induction of enzyme in certain tumor cell lines in-vitro, but does not disclose relative enzyme levels in related normal tissue in-vivo.

Therefore, there exists a need for the development of a method for monitoring the clinical effectiveness of a polyamine analog anticancer agents, including the bis-ethyl spermine analogs, which have potent anticancer activity against certain cancers such as melanoma. In addition, a method for determining therapeutic effectiveness of treatment, and for predicting the sensitivity to treatment of an individual's tumor with analogs, such as a bis-ethyl spermine analog, is desired. Such methods will greatly facilitate the identification of, and chemotherapeutic treatment of individuals bearing tumors sensitive to analogs that effect induction of SSAT such as bis-ethyl spermine analogs.

2. Summary of the Invention

A primary object of the invention is to provide a method for predicting the responsiveness of an individual's tumor to treatment with polyamine analogs that effect SSAT induction such as bis-ethyl spermine analogs.

Another object of the invention is to provide a method for monitoring the therapeutic effectiveness of treatment, using this class of polyamine analogs, of an individual bearing tumors sensitive to this class of analogs.

A further object of the present invention is to provide a method for determining a therapeutic regimen and treatment schedule for individuals undergoing chemotherapy with polyamine analogs that effect induction of SSAT.

In summary, the above is accomplished by providing a method wherein the levels of SSAT are measured in an individual's tumor cells which have been previously exposed to treatment with a polyamine analog. Induction of SSAT may be used as indica of sensitivity to, therapeutic effectiveness of, and to determine clinically efficacious amounts of, polyamine analogs.

3. Brief Description of the Drawings:

A more complete appreciation of the invention, and its many attendant advantages thereof, and a better understanding of its features may follow by referring to the detailed description in connection with the accompanying figures, wherein:

FIG. 1 is a diagram depicting the chemical relationship between spermine and a polyamine analog comprising the bis-ethyl spermine analog N^1,$N^{1,2}$-Bis(ethyl) spermine.

FIG. 2 is a diagram depicting the chemical relationship between three selected bis-ethyl spermine analogs which differentially induce SSAT.

FIG. 3 is a bar graph depicting the in-vivo induction of SSAT activity following bis-ethyl spermine analog treatment.

FIG. 4 is a diagram depicting a biochemical pathway involving spermine and derivatives thereof.

4. Detailed Description

The method of using the induction of spermidine/spermine N^1-acetyltransferase as a prognostic marker or as a tumor response marker, in relation to treatment of tumors with polyamine analogs, includes the direct measurement of SSAT activity in tumor biopsies, and also includes- measurement of other determinants relating to SSAT levels such as detection of metabolic products of SSAT as found in tissue or serum; detection in tissue of amplified SSAT-specific messenger RNA (m-RNA) transcripts, measurements of corresponding

changes in levels of enzymes affected by the induction of SSAT activity, and detection and quantification in tissue of increased amounts of the enzyme protein itself such as by enzyme-linked immunosorbent assay (ELISA).

The three bis-ethyl spermine analogs, chosen as a representative panel of polyamine analogs for use to perform relevant in-vivo studies, include N^1,N^{12}-bis(ethyl)spermine (BESPM); N^1,N^{11}-bis-(ethyl)norspermine (BENSPM) and N^1,N^{14}-bis-(ethyl)homospermine (BEHSPM). All three similarly suppress ornithine and S-adenosyl-methionine decarboxylase levels, but differentially induce SSAT levels in-vitro (Porter et al. Cancer Research 51:3715-3720, 1991). The chemical relationship between BESPM and the two related compounds, BEHSPM and BENSPM, is shown in Figure 2.

4.1 SSAT Induction in-vivo and Correlation in-vivo of Antitumor Activity of N,N'-Bis(ethyl)spermine Analogs with SSAT Induction

The in-vivo antitumor activities of BESPM, BEHSPM, and BENSPM, were compared against MALME-3 human melanoma xenografts in mice. Cultured MALME-3 melanoma cells were first passaged several times in female HSD nude athymic mice. Fragments of resultant tumor were implanted into mice via subcutaneous trocar implantation. Mice of different treatment groups were treated with one of the three bis-ethyl spermine analogs BESPM, BEHSPM, and BENSPM. Tumor growth inhibition was monitored by parameters including tumor volume. Against the MALME-3 human melanoma xenografts in mice, BESPM displayed significant antitumor activity evidenced by the suppression of tumor growth for an additional 30 days following cessation of treatment. By comparison, BEHSPM was less effective than BESPM, in that BEHSPM suppressed tumor growth for 18 days after cessation of treatment. BENSPM was the most effective of the three in that it suppressed tumor growth for 40 days after the cessation of treatment. The antitumor activity for these three analogs correlated with their ability to induce SSAT activity in the same tumor cells growing in-vitro (Table 1). It was also noted that retreatment 2 weeks later with BENSPM resulted in an apparent curing of about 20% of the MALME-3 xenografts. This antitumor activity exhibited is sufficiently significant to warrant clinical testing of polyamine analogs that effect SSAT induction, and in particular, BENSPM.

Studies were performed using BENSPM treatment of MALME-3 human melanoma xenografts to determine if there exists a correlation between antitumor activity and SSAT induction in-vivo. SSAT activity was measured using cell extracts obtained by sonication, in 5 mM N-2 hydroxypiperazine-N^2-ethanesulfonic acid (pH 7.2) containing 1 mM dithiothreitol, of tumor biopsy from treated mice, and tumor biopsy from control mice. The cytosolic extract resulting from a 1 hour centrifugation at 35,000 rpm in a Spinco 40 rotor was used as the source of the enzyme for the assay of SSAT activity. The cytosolic extract was incubated with 10 umol HEPES buffer, pH7.8, 0.15 nmol spermidine, and 0.5 nmol [1-^{14}C] acetylCoenzyme A, in a final volume of 50 ul, for 5 minutes at 37°C. The reaction was stopped by chilling, the addition of 20 ul of 0.5 M $NH_2OH{\cdot}Cl$, and heating in a boiling water bath for 3 minutes. After centrifugation to remove precipitated protein, 50 ul of the reaction was spotted on a disc of P-81 phosphocellulose and counted for radioactivity. Protein concentration was also measured so that enzyme activity was expressed as picomoles of acetylspermine synthesized per minute per milligram of protein.

SSAT activity, in tumors taken 16 hours following the final BENSPM injection, was elevated to approximately 13,700 pmol/min/mg as compared with the activity of 75 pmol/min/mg in control tumors (Table 2). Also kidney and liver SSAT activities in the mice treated with BENSPM were found to be increased from basal levels of approximately 10 pmol/min/mg to 1255 and 320 pmol/min/mg, respectively. BENSPM-treated tumors had polyamine pools which were almost totally depleted at this time. Two weeks after treatment, the tumor from MALME-3 human melanoma xenografts in BENSPM-treated mice contained SSAT levels of 3,040 pmol/min/mg as compared to 260 and 75 pmol/min/mg for kidney and liver, respectively. Note that ODC and SAMDC levels were not suppressed, suggesting that polyamine depletion may be due totally to SSAT induction. Thus, the latter may be indirectly responsible for growth inhibition and, therefore, a determinant of drug action in-vivo.

In the absence of other major biochemical perturbations, the dramatically increased SSAT levels in treated tumor seem to suggest a role for the enzyme in the initiating growth inhibition during treatment and in sustaining the effect after treatment (Figure 3). Since polyamine pools at the two week period (Table 3) are not characteristic of polyamine inhibitor-induced growth inhibition (i.e. pools are not markedly depleted), SSAT may mediate the antiproliferative response by other means. These other means may include rapid acetylation of all unbound spermidine and spermine thus eliminating availability of free polyamines; an excessive accumulation of N^1-acetylspermidine; depletion of acetylCoenzyme-A pools (co-factor for SSAT as shown in Fig. 4); inappropriate acetylation of and possible inactivation of critical molecules or receptors; or any combination of the above.

Table 1 - Comparison of BESPM Homolog Effects

Homolog	SSAT Induction*	Regrowth delay**	%T/C***
BEHSPM	~1,000	14	43
BESPM	~10,000	27	34
BENSPM	~60,000	42	<10

* (pmol/min/mg) in cultured MALME-3 cells treated for 48 hours with 10uM homolog.

** Time (in days) required following treatment for median tumor volume to reach 200 mm^3.

*** %T/C is treated tumor volume minus pretreatment volume divided by control tumor volume minus pretreatment volume at day 95 (when control tumor volume = $1000mm^3$) times 100.

Table 2. Effect of BENSPM on MALME-3 Melanoma and Host Tissue Polyamine-Related Enzymes

Tissue	BENSPM Treated*	Enzyme Activities ODC (nmol/hr/mg)	SAMDC (nmol/hr/mg)	SSAI (pmol/min/mg)
16 Hrs Post-treatment:				
Tumor	–	0.23/0.13**	0.35/0.28	67/83
	+	0.21/0.16	0.42/0.41	11,490/15,930
Kidney	–	0.16/0.14	0.25/0.36	6/8
	+	0.18/0.21	0.56/0.47	1616/895
Liver	–	0.11/0.08	1.05/1.68	11/8
	+	0.21/0.15	1.23/1.03	347/294
2 Wks Post-treatment:				
Tumor	–	0.17/0.20	0.62/0.65	70/75
	+	0.28/0.99	0.96/2.14	2810/3275
Kidney	–	0.14/0.13	0.26/0.33	5/3
	+	0.13/0.13	0.49/0.37	272/248
Liver	–	0.06/0.06	1.27/1.32	6/5
	+	0.06/0.08	1.01/1.14	90/60

*40 mg/kg 3x/day x6 days.

**Duplicate mice with each value representing the mean of duplicate assays.

Table 3. *Effect of BENSPM on MALME-3 Melanoma and Host Tissue Polyamine and Analog Pools*

Tissue	BENSPM Treated*	PUT	SPD	SPM	BENSPM
		(pmol/mg protein)			
16 Hrs Post-treatment:					
Tumor	-	60/20**	320/290	450/380	-
	+	20/20	50/20	40/30	1020/1535++
Kidney	-	20/20	240/260	540/440	-
	+	100/40	100/100	260/250	860/1705++
Liver	-	20/20	570/460	610/500	-
	+	90/100	80/90	170/200	1180/1470++
2 Hks Post-treatment:					
Tumor	-	20/50	330/270	330/360	-
	+	370/290	560/160+	200/80	620/470
Kidney	-	20/30	340/320	530/520	-
	+	20/20	210/210	360/370	90/150
Liver	-	20/20	490/660	660/760	-
	+	20/20	530/530	400/330	40/40

*BENSPM 40 mg/kg 3x/day x6 days.

**Duplicate mice with each value representing the mean of duplicate determinations.

+also contained high levels of N-acetylspermidine (240/180 pmol/mg protein).

++also contained a peak which was similar in height to the SPM peak of this tissue and which was presumed by location to be a monoethyl metabolite peak of BENSPM.

5. Preferred Embodiments

The following examples are directed to the measurement of determinants related to the in-vivo induction of SSAT, subsequent to polyamine analog treatment of tumor, which may be used as indica of sensitivity to, therapeutic effectiveness of, and to determine clinically efficacious amounts of, polyamine analogs.

5.1 Use of SSAT as a Predictive Assay for Bis-ethyl Spermine Analog Therapy

Because the the level of induction of SSAT, in response to polyamine analogs such as bis-ethyl spermine analogs, is extremely variable among human tumor types including human melanoma, quantitation of SSAT superinduction could be used in a pre-therapy test for predicting responsiveness of that particular human tumor type to the bis-ethyl spermine analog to be administered. Clinical trials, to establish a range of SSAT induction responses among patients, could be used to draw a correlation between SSAT induction and clinical response to treatment.

Example 1

Tumor biopsies or surgical specimens containg tumor may be disaggregated, and introduced into cell culture; or alternatively, grown in culture as explants. Treatment with the polyamine analog such as a bis-ethyl spermine analog is then initiated and continued for up to four cell doublings without medium change. The treated cells are then prepared as cell suspensions for quantitation of SSAT induction.

Embodiment A: SSAT induction can be quantitated by assaying for enzyme activity. Cell extracts of the treated cultured tumor cells may be obtained by sonication, in 5 mM N-2 hydroxypiperazine-N^2-ethanesulfonic acid (pH 7.2) containing 1 mM dithiothreitol. The cytosolic extract resulting from a 1 hour centrifugation at 35,000 rpm in a Spinco 40 rotor is used as the source of the enzyme for the assay of SSAT activity. The cytosolic extract is incubated with 10 umol HEPES buffer, pH7.8, 0.15 nmol spermidine, and 0.5 nmol [1-^{14}C] acetyl-Coenzyme A, in a final volume of 50 ul, for 5 minutes at 37°C. The reaction is stopped by chilling, the addition of 20 ul of 0.5 M $NH_2OH{\cdot}Cl$, and heating in a boiling water bath for 3 minutes. After centrifugation to remove precipitated protein, 50 ul of the reaction is spotted on a disc of P-81 phosphocellulose and counted for radioactivity. Protein concentration is also measured so that enzyme activity was expressed as picomoles of acetylspermine synthesized per minute per milligram of protein.

Embodiment B: SSAT induction can be quantitated by using a specific antibody to assay for the physical presence of SSAT. In one mode of this embodiment, SSAT may be used as an antigen in immunoassays designed to detect and quantificate SSAT induction. A cell extract or cytosolic extract is prepared as according to the methods in Embodiment A. The detection of SSAT as an antigen in prepared extracts includes any immunoassay system known in the art including, but not limited to: radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), "sandwich" assays, precipitin reactions, agglutination assays, and fluorescent immunoassays.

In another mode of this embodiment, following treatment with a polyamine analog, whole cells can be assayed for the physical presence of SSAT in culture as a monolayer after which an in-situ immunocytochemical assay can be performed to detect the presence of an induction of SSAT. This may be quantitated by visual inspection of cell monolayers using a fluorescent microscope, or automatically by fluorescence flow cytometry of a cell suspension prepared from the cell monolayer.

Embodiment C: The basis for the induction of SSAT activity is an induction of enzyme protein related to the combined effects of increased enzyme-specific mRNA (messenger RNA) accumulation, and decreased enzyme protein degradation. Therefore, an alternative to assaying enzyme activity or protein would be to assay for an increase in SSAT-specific m-RNA transcripts. m-RNA can be purified from cell extracts and then subjected to enzymatic amplification to obtain sufficient quantities for analysis and detection. Enzymatic amplification techniques which could be employed include those known in the art such as PCR (polymerase chain reaction), QB replicase, and NASBA (nucleic acid sequence-based amplification). Detection techniques includes systems known in the art including, but not limited to, agarose gel electrophoresis and Northern blotting; fluorescence-based hybridization assays; chemilumines-cence-based hybridization assays; and capture hybridization microtiter assays.

Embodiment D: The above procedures and methods of Embodiments A-C would be utilized to measure other indica related to SSAT induction rather than measuring SSAT-specific enzyme activity, protein, or mRNA transcripts. These indica include decreases in the SSAT co-factor acetylCoenzyme A, or increases in SSAT products such as N^1-acetylspermidine and N^1-acetylspermine. Also, mutated or variants of the gene encoding SSAT may be indicative of the potential for SSAT induction.

Example 2

Patients having tumor types suspected of being responsive to one or more polyamine analogs, such as bis-ethyl spermine analogs, would be administered a single dose of the analog. Subsequent to this treatment, tumor tissue from the patient would be obtained by biopsy and the tissue may then be subjected to one or more of the methods and procedures outlined in Example 1, Embodiments A-D, for detecting an induction of SSAT.

5.2 Use of SSAT as a Tumor Response Marker for Bis-ethyl Spermine Analog Treatment

Immediately following bis-ethyl spermine analog treatment, occurs a dramatic increase in SSAT activity. More importantly, the high levels of SSAT activity continue during the sustained growth inhibition following bis-ethyl spermine analog treatment, as exemplified in Figure 3 with BENSPM treatment. Therefore, the SSAT induction response may serve as a tumor response marker for specifically monitoring the therapeutic effectiveness of bis-ethyl spermine analog treatment, and for determining a dosage regimen and treatment schedule for an individual patient having tumor responsive to bis-ethyl spermine analogs. An indicator useful in determining a dosage regimen and treatment schedule would be particularly desirable in cases where a patient develops adverse side effects as a result of bis-ethyl spermine analog treatment.

Embodiment A: Following treatment with a bis-ethyl spermine analog, and when tumor is conveniently accessible, tumor may be biopsied from the patient. SSAT induction can be quantitated by assaying for enzyme activity. Cell extracts of the bi6psied tumor cells may be prepared, and enzyme activity measured, in accordance with the methods and procedures of Example 1, Embodiment A.

Embodiment B: Alternatively, SSAT induction may be quantitated from the biopsied tumor cell extract by using a specific antibody to assay for the physical presence of SSAT according to the methods and procedures of Example 1, Embodiment B. In one mode of this embodiment, SSAT may be used as an antigen in immunoassays designed to detect and quantificate SSAT induction. The detection of SSAT as an antigen in prepared extracts includes any immunoassay system known in the art including, but not limited to: radioimmunoassays; enzyme-linked immunosorbent assays (ELISA); "sandwich" assays; precipitin reactions; agglutination assays; and fluorescent immunoassays.

In another mode of this embodiment, an in-situ cytochemical assay can be performed directly on a histological preparation of the tumor biopsy to detect the presence of an induction of SSAT.

Embodiment C: The basis for the induction of SSAT activity is an induction of enzyme protein related to the combined effects of increased enzyme-specific mRNA (messenger RNA) accumulation, and decreased enzyme protein degradation. Therefore, an alternative to assaying enzyme activity or protein would be to assay for an increase in SSAT-specific m-RNA transcripts as in accordance with the methods and procedures of Example 1, Embodiment C. Systems useful for detecting m-RNA transcripts may be selected from the group consisting of agarose gel electrophoresis and Northern blotting; fluorescence-based hybridization assays; chemiluminescence-based hybridization assays; and capture hybridization microtiter assays.

In another embodiment, the physical presence of SSAT specific mRNA levels can be detected by performing in-situ hybridization directly on a histological preparation of the tumor biopsy.

Embodiment D: The above procedures and methods of Embodiments A-C would be utilized to measure other indica related to SSAT induction rather than measuring SSAT-specific enzyme activity, protein, or mRNA transcripts. These indica include decreases in SSAT co-factor acetylCoenzyme A, or increases in SSAT products such as N^1-acetylspermidine and N^1-acetylspermine. Also, mutated or variants of the gene encoding SSAT may be indicative of the potential for SSAT induction.

Embodiment E: Following treatment with a bis-ethyl spermine analog, as an alternative to performing a tumor biopsy from the patient, a blood sample may be drawn, or a urine sample collected. Serum, red blood cell (rbc), or urine levels of SSAT-related products may be selected from the group consisting of N^1-acetylspermidine and N^1-acetylspermine. Serum, rbc, or urine levels of these products may be determined using quantitative chromatographic techniques known in the art, such as by HPLC (high pressure liquid chromatography); or an immunoassay system known in the art selected from the group consisting of radioimmunoassays, enzyme-linked immunosorbent assays (ELISA), "sandwich" assays, precipitin reactions, agglutination assays, and fluorescent immunoassays.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, various modifications will become apparent to persons skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to be included within the spirit of this application and within the scope of the appended claims.

Claims

1. A method for predicting the responsiveness of a mammalian tumor type to chemotherapy with an anticancer agent comprising a polyamine analog that induces spermidine/spermine N^1-acetyltransferase, which comprises the steps of:
   (a) performing a biopsy to obtain tumor cells;
   (b) introducing said tumor cells or tumor tissue in culture;

(c) administering a therapeutically effective amount of said polyamine analog to the tumor cells in culture; and
(d) detecting the level of induction of spermidine/spermine N^1-acetyltransferase in the tumor cells exposed to said polyamine analog.

2. The method of claim 1, wherein the polyamine analog is a bis-ethyl spermine analog selected from the group consisting of N^1,N^{12}-bis(ethyl)spermine, N^1,N^{11}-bis(ethyl)norspermine, N^1,N^{14}-bis (ethyl)homospermine, or a combination thereof.

3. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by quantifying spermidine/spermine N^1-acetyltransferase activity.

4. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for the physical presence of spermidine/spermine N^1-acetyltransferase protein.

5. The method of claim 4, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for by using spermidine/spermine N^1-acetyltransferase protein as an antigen to be detected in an immunoassay selected from the group consisting of a radioimmunoassay, enzyme-linked immunosorbent assay, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent-based immunoassay, and chemiluminescence-based immunoassay.

6. The method of claim 4, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for in intact cells by an in-situ cytochemical assay specific for spermidine/spermine N^1-acetyltransferase.

7. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/spermine N^1-acetyltransferase m-RNA using a detection technique selected from the group consisting of agarose gel electrophoresis and Northern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

8. The method of claim 7, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is assayed for in intact cells by in-situ hybridization.

9. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by detecting mutations or variations in the gene encoding spermidine/spermine N^1-acetyltransferase using a detection technique selected from the group consisting of agarose gel electrophoresis and Southern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

10. The method of claim 7, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is first enzymatically amplified.

11. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for a polyamine derivative selected from the group consisting of N^1-acetylspermidine, and N^1-acetylspermine.

12. The method of claim 1, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/ spermine N^1-acetyltransferase cofactor acetylCoenzyme A.

13. A method.for predicting the responsiveness of a mammalian tumor type to chemotherapy with an anticancer agent comprising a polyamine analog that induces spermidine/spermine N^1-acetyltransferase, which comprises the steps of:
(a) administering a therapeutically effective amount of said polyamine analog to the mammal;
(b) performing a biopsy to obtain tumor cells; and
(c) detecting the level of induction of spermidine/spermine N^1-acetyltransferase in the tumor cells exposed to said polyamine analog.

14. The method of claim 13, wherein the polyamine analog is a bis-ethyl spermine analog selected from the group consisting of N^1,N^{12}-bis(ethyl)spermine, N^1,N^{11}-bis(ethyl)norspermine, N^1,N^{14}-bis

(ethyl)homospermine, or a combination thereof.

15. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by quantifying spermidine/spermine N^1-acetyltransferase activity.

16. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for the physical presence of spermidine/spermine N^1-acetyltransferase protein.

17. The method of claim 16, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for by using spermidine/spermine N^1-acetyltransferase protein as an antigen to be detected in an immunoassay selected from the group consisting of a radioimmunoassay, enzyme-linked immunosorbent assay, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent-based immunoassay, and chemiluminescence-based immunoassay.

18. The method of claim 16, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for in intact cells by an in-situ cytochemical assay specific for spermidine/spermine N^1-acetyltransferase.

19. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/spermine N^1-acetyltransferase m-RNA directly from the tumor cells obtained by biopsy using a detection technique selected from the group consisting of agarose gel electrophoresis and Northern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

20. The method of claim 19, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is assayed for in intact cells by in-situ hybridization.

21. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by detecting mutations or variations in the gene encoding spermidine/spermine N^1-acetyltransferase using a detection technique selected from the group consisting of agarose gel electrophoresis and Southern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

22. The method of claim 19, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is first enzymatically amplified.

23. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for a polyamine derivative selected from the group consisting of N^1-acetylspermidine, and N^1-acetylspermine.

24. The method of claim 13, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/ spermine N^1-acetyltransferase cofactor acetylCoenzyme A.

25. A method useful for monitoring the therapeutic effectiveness of, and determining a dosage regimen and treatment schedule for, chemotherapy in a tumor-bearing mammal with an anticancer agent comprising a polyamine analog that induces spermidine/spermine N^1-acetyltransferase, which comprises the steps of:

(a) administering an therapeutically effective amount of said polyamine analog to a mammal;
(b) performing a biopsy from the mammal to obtain tumor cells;
(c) detecting the level of induction of spermidine/spermine N^1-acetyltransferase in the treated tumor cells.

26. The method of claim 25, wherein said polyamine analog comprises a bis-ethyl spermine analog selected from the group consisting of N^1,N^{12}-bis(ethyl)spermine, N^1,N^{11}-bis(ethyl)norspermine, N^1,N^{14}-bis(ethyl)homospermine, or a combination thereof.

27. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by quantifying spermidine/spermine N^1-acetyltransferase activity.

28. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured

by assaying for the physical presence of spermidine/spermine N^1-acetyltransferase protein.

29. The method of claim 28, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for by using spermidine/spermine N^1-acetyltransferase protein as an antigen to be detected in an immunoassay selected from the group consisting of a radioimmunoassay, enzyme-linked immunosorbent assay, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent-based immunoassay, and chemiluminescence-based immunoassay.

30. The method of claim 28, wherein the physical presence of spermidine/spermine N^1-acetyltransferase protein is assayed for by an in-situ cytochemical assay specific for spermidine/spermine N^1-acetyltransferase.

31. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/spermine N^1-acetyltransferase m-RNA directly from the tumor cells obtained by biopsy using a detection technique selected from the group consisting of agarose gel electrophoresis and Northern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

32. The method of claim 31, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is assayed for in intact cells by in-situ hybridization.

33. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying mutations or variations in the gene encoding spermidine/spermine N^1-acetyltrans-ferase using a detection technique selected from the group consisting of agarose gel electrophoresis and Southern blotting, fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

34. The method of claim 31, wherein the spermidine/spermine N^1-acetyltransferase m-RNA is first enzymatically amplified.

35. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for a polyamine derivative selected from the group consisting of N^1-acetylspermidine, and N^1-acetylspermine.

36. The method of claim 25, wherein the induction of spermidine/spermine N^1-acetyltransferase is measured by assaying for spermidine/ spermine N^1-acetyltransferase cofactor acetylCoenzyme A.

37. A method useful for monitoring the therapeutic effectiveness of, and determining a dosage regimen and treatment schedule for, chemotherapy in a tumor-bearing mammal with an anticancer agent comprising a polyamine analog that induces spermidine/spermine N^1-acetyltransferase, which comprises the steps of:

(a) administering an therapeutically effective amount of said polyamine analog to a mammal;
(b) removing body fluid from said mammal; and
(c) detecting the level of induction of spermidine/spermine N^1-acetyltransferase by measuring a polyamine derivative selected from the group consisting of N^1-acetylspermidine, N^1-acetylspermine, or a combination thereof.

38. The method of claim 37, wherein said polyamine analog comprises a bis-ethyl spermine analog selected from the group consisting of N^1,N^{12}-bis(ethyl)spermine, N^1,N^{11}-bis(ethyl)norspermine N^1,N^{14}-bis(ethyl)homospermine, or a combination thereof.

39. The method of claim 37, wherein said body fluid is selected from the group consisting of blood, urine, or a combination thereof.

40. A diagnostic kit for quantifying spermidine/spermine N^1-acetyltransferase activity.

41. A diagnostic kit for detecting the physical presence of spermidine/spermine N^1-acetyltransferase protein as an antigen in an immunoassay selected from the group consisting of a radioimmunoassay, enzyme-linked immunosorbent assay, "sandwich" assay, precipitin reaction, agglutination assay, fluorescent-based immunoassay, and chemiluminescence-based immunoassay.

42. A diagnostic kit for detecting spermidine/spermine N^1-acetyltransferase m-RNA using a detection technique selected from the group consisting of fluorescence-based hybridization assay, chemiluminescence-based hybridization assay, and capture hybridization microtiter assay.

43. A diagnostic kit for detecting a polyamine derivative selected from the group consisting of N^1-acetylspermidine, and N^1-acetylspermine.

44. A diagnostic kit for detecting spermidine/ spermine N^1-acetyltransferase cofactor acetylCoenzyme A.

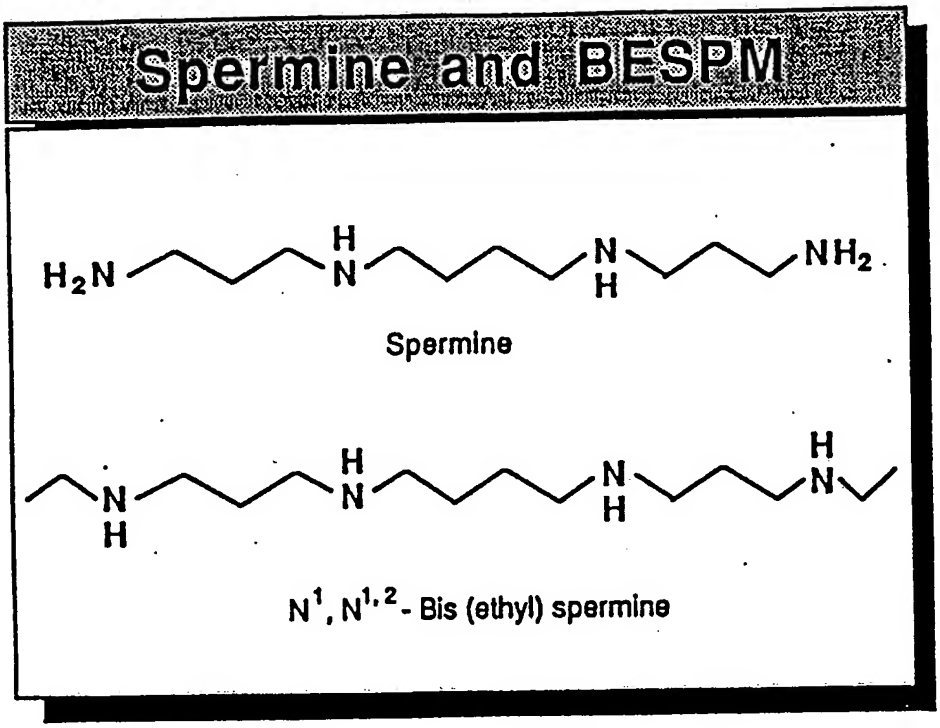
Spermine and BESPM
H2N
H
N
N
H
NH2
Spermine
N
H
H
N
N
H
H
N
N1, N1,2 - Bis (ethyl) spermine

Figure 1

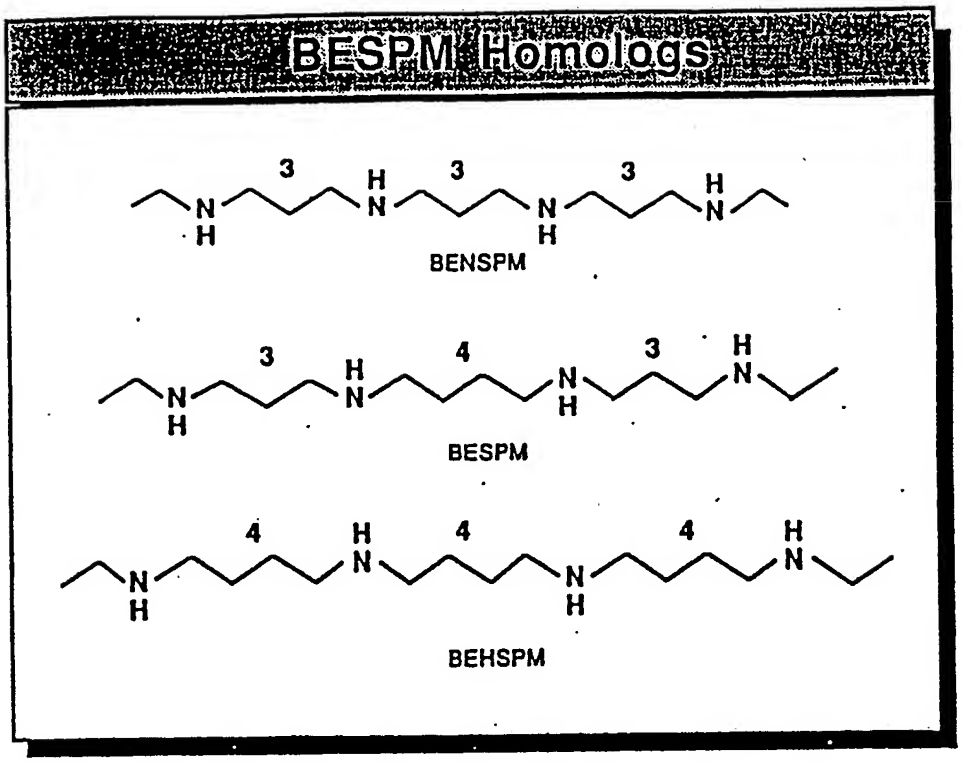

Figure 2

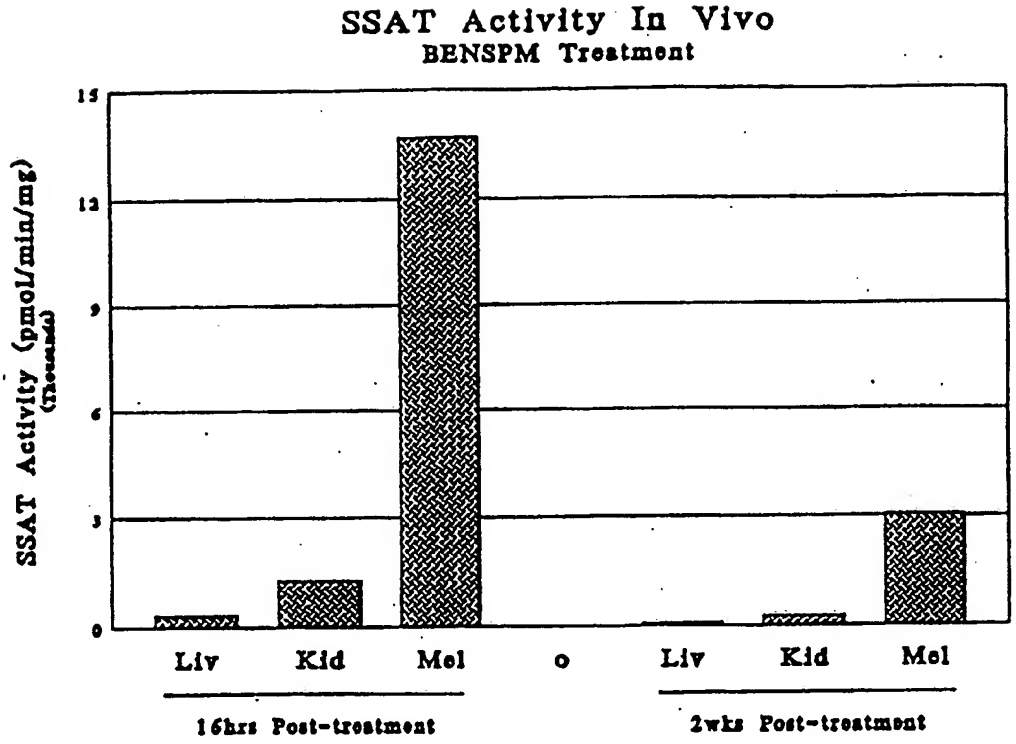
SSAT Activity In Vivo
BENSPM Treatment
SSAT Activity (pmol/min/mg)
15
12
9
6
3
0
Liv
Kid
Mel
o
Liv
Kid
Mel
16hrs Post-treatment
2wks Post-treatment

Figure 3

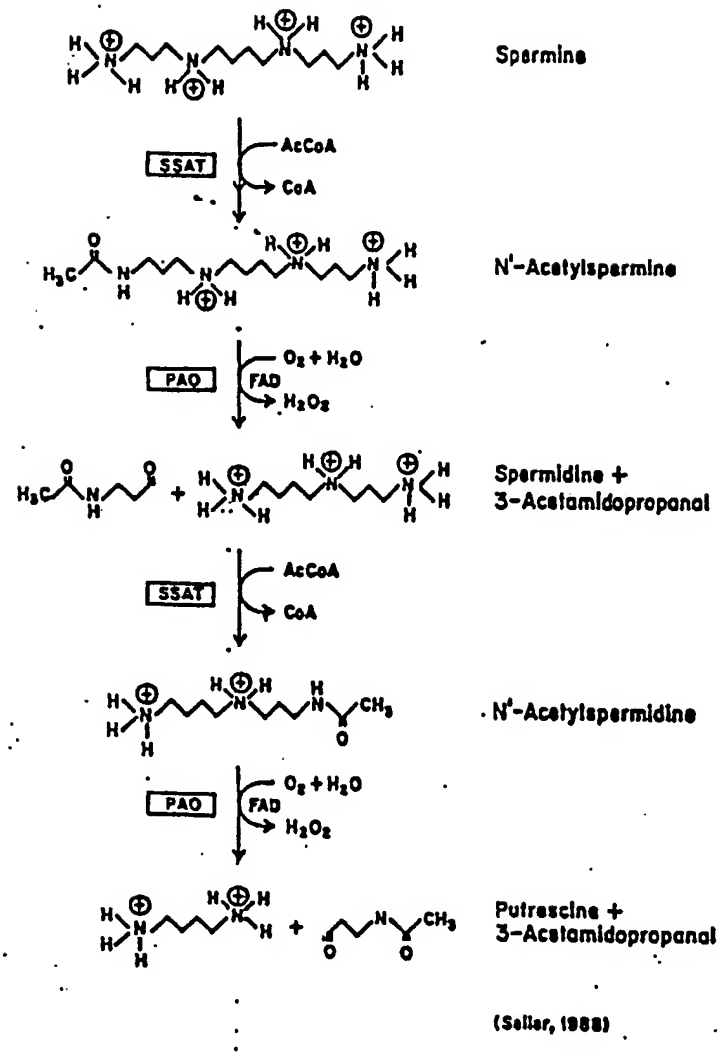
Spermine
SSAT
AcCoA
CoA
N¹-Acetylspermine
PAO
O₂ + H₂O
FAD
H₂O₂
Spermidine +
3-Acetamidopropanal
SSAT
AcCoA
CoA
N¹-Acetylspermidine
PAO
O₂ + H₂O
FAD
H₂O₂
Putrescine +
3-Acetamidopropanal
(Seiler, 1988)

Figure 4

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(54) Methods for the use of spermidine/spermine N'-acetyltransferase as a progonostic indicator and/or a tumor response marker.

(57) Disclosed is a method that relates to the measurement of determinants related to the in-vivo induction of spermidine/ spermine N[1]-acetyltransferase (SSAT) , subsequent to treatment with polyamine analogs that induce SSAT such as bis-ethyl spermine analogs, in tumor types responsive to this class of analogs. The method comprises the measurement of one or more SSAT-specific determinants that include SSAT enzyme activity, SSAT enzyme protein, and SSAT m-RNA transcripts. Alternatively, other determinants related to the SSAT induction may be measured. Such determinants include SSAT co-factor acetylCoenzyme A, and SSAT products N[1]-acetylspermidine and N[1]-acetylspermine. Measurements of these determinants may be useful as prognostic indicia and tumor response markers to evaluate the clinical effectiveness of anticancer agents comprising polyamine analogs, such as bis-ethyl spermine analogs, that induce SSAT activity.

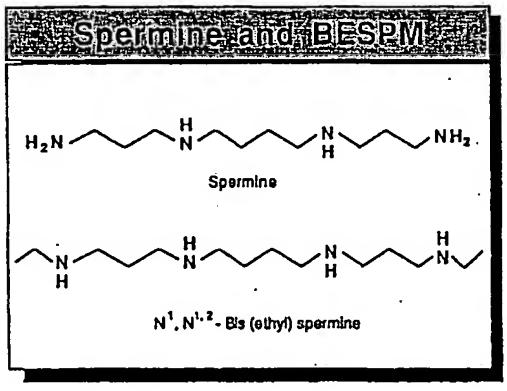

Figure 1

EP 0 568 338 A3

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 93 30 3312

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
D,X	CANCER RESEARCH, vol.51, no.14, 15 July 1991, CHICAGO, ILL. pages 3715 - 3720 PORTER ET AL. 'Correlations between polyamine analogue-induced increases in spermidine/spermine N1-acetyltransferase activity, ...' * the whole document * ---	1-5,7, 10,11, 40-43	C12Q1/48 G01N33/573 G01N33/574 G01N33/50 //A61K31/13, A61K31/16
D,X	BIOCHEMICAL JOURNAL, vol.270, no.3, 1990, LONDON pages 615 - 620 CASERO ET AL. 'High specific induction of spermidine/spermine N1-acetyltransferase ....' * the whole document * --- -/--	1-5,7, 12, 40-42,44	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C12Q
G01N

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely: 1-12,40-44

Claims searched incompletely:

Claims not searched: 13-39

Reason for the limitation of the search:

Art. 52(4) EPC: Method for treatment/diagnosis practised on the human or animal body.

Place of search	Date of completion of the search	Examiner
BERLIN	31 January 1994	Ceder, O

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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PARTIAL EUROPEAN SEARCH REPORT

Application Number EP 93 30 3312

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	CANCER RESEARCH, vol.49, no.14, 15 July 1989, CHICAGO, ILL pages 3829 - 3833 CASERO ET AL. 'Differential induction of spermidine/spermine N1-acetyltransferase ....' * abstract * * page 3829, right column - page 3830, left column * ---	1-4,11, 12,40,44	
D,X	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol.284, no.2, 1 February 1991, NEW YORK, NY pages 238 - 244 LIBBY ET AL. 'Characterization of human spermidine/spermine N1-acetyltransferase purified from cultured melanoma cells' * abstract * * page 240, left column * * page 241, right column * ---	1-4,11, 40,43	TECHNICAL FIELDS SEARCHED (Int.Cl.5)
P,X	CANCER RESEARCH, vol.52, 1 October 1992, CHICAGO, ILL. pages 5359 - 5363 CASERO ET AL. 'Steady-state messenger RNA ....' * the whole document * -----	1-4,7, 40,43	

EPO FORM 1503 03.82 (P04C03)